Bioresource Technology 139 (2013) 272–279

Contents lists available at SciVerse ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Consolidated bioprocessing of untreated switchgrass to hydrogen by the extreme thermophile *Caldicellulosiruptor saccharolyticus* DSM 8903

Suvarna Talluri [a], Subramanian Mohan Raj [a,1], Lew Paul Christopher [a,b,*]

[a] Center for Bioprocessing Research and Development, South Dakota School of Mines and Technology, Rapid City, SD 57701, USA
[b] Department of Civil and Environmental Engineering, South Dakota School of Mines and Technology, Rapid City, SD 57701, USA

HIGHLIGHTS

- *Caldicellulosiruptor saccharolyticus* fermented untreated switchgrass and MCC to H_2.
- The extreme thermophile produced the maximum theoretical yield of 4 mol H_2/mol glucose.
- Pretreatment, enzyme production, hydrolysis, fermentation were combined in one step.
- *C. saccharolyticus* DSM 8903 is a promising candidate for consolidated bioprocessing.
- Potential for cost savings from capital and operating expenditures could exceed 50%.

ARTICLE INFO

Article history:
Received 8 January 2013
Received in revised form 1 April 2013
Accepted 2 April 2013
Available online 18 April 2013

Keywords:
Hydrogen
Untreated switchgrass
Caldicellulosiruptor saccharolyticus DSM 8903
Extreme thermophiles
Consolidated bioprocessing

ABSTRACT

The abilities of the extreme thermophilic bacterium *Caldicellulosiruptor saccharolyticus* DSM 8903 to ferment switchgrass (SWG), microcrystalline cellulose (MCC) and glucose to hydrogen (H_2) in one-step were examined. Hydrogen production from glucose reached the theoretical maximum for dark fermentation of 4 mol H_2/mol glucose. The H_2 yield on MCC and SWG after 6 days of fermentation was 23.2 mmol H_2/L or 9.4 mmol H_2/g MCC and 14.3 mmol H_2/L or 11.2 mmol H_2/g SWG, respectively. The rate of H_2 formation however was higher on MCC (0.7 mmol/L h) than SWG (0.1 mmol/L h). *C. saccharolyticus* DSM 8903 was able to produce H_2 directly from mechanically-comminuted SWG without any physicochemical or biological pretreatment. Combining four processing steps (pretreatment, enzyme production, saccharification and fermentation) into a single biorefinery operation makes *C. saccharolyticus* DSM 8903 a promising candidate for consolidated bioprocessing (CBP) of lignocellulosic biomass.

1. Introduction

Lignocellulosic biomass is a renewable and abundant resource that is available in the U.S. at nearly 1.3 billion tons per year as a low-cost feedstock for production of biofuels (Zheng et al., 2012). However, due to the intimate association of polysaccharides and lignin, the lignocellulosic matrix is recalcitrant to enzymatic breakdown and pretreatment is usually required to facilitate the release of fermentable sugars from plant biomass. The conversion of cellulose and hemicellulose, in their cell wall-bound form, into fermentable sugars in a cost-effective way presents a major challenge to the commercialization of cellulosic biofuels. Several thermal, chemical, physical and biochemical pretreatment methods have been proposed to convert lignocellulosic biomass into fermentable sugars, but none of these approaches have been commercialized due to two major reasons: (1) high costs of pretreatment processes; and (2) generation of microbial growth inhibitory compounds such as phenols, organic acids, furfurals and/or hydroxylmethyl furfurals in most thermal and acid pretreatment processes (Yang and Wyman, 2008). The pretreatment process is usually energy-intensive and/or requires the use of hazardous chemicals and expensive enzymes for the hydrolysis step. Economic analyses have revealed that the greatest fraction of projected costs of nearly 40% is associated with the release of fermentable sugars from

* Corresponding author at: Center for Bioprocessing Research and Development, South Dakota School of Mines and Technology, Rapid City, SD 57701, USA. Tel.: +1 605 394 3385; fax: +1 605 394 1686.
E-mail address: lew.christopher@sdsmt.edu (L.P. Christopher).
[1] Present address: Center for Research and Development, PRIST University, Thanjavur, TN 613 403, India.

0960-8524
http://dx.doi.org/10.1016/j.biortech.2013.04.005

biomass by the combined operations of pretreatment, enzyme production, and enzymatic hydrolysis, with pretreatment responsible for almost half of this total (Wooley et al., 1999). With 20% of the total costs, pretreatment is projected to be the single, most expensive processing and rate-limiting step (Yang and Wyman, 2008) of the overall H_2 production process. Hence, the key to the success of cellulosic H_2 relies on the development of breakthrough technologies allowing effective and low-cost saccharification and fermentation of lignocellulosic feedstock.

A significant step toward commercialization of cellulose-derived biofuels including H_2 is presented with the CBP process configuration which combines three major biomass processing steps – enzyme production, enzyme hydrolysis of the biomass carbohydrate components (cellulose and hemicellulose), and fermentation of the hexose and pentose sugars – into a single step (Lynd et al., 2005; Bielen et al., 2013). This integrated process has been proposed as the most cost-efficient and ultimate industrial configuration for low cost hydrolysis and fermentation of cellulosic biomass. CBP has an outstanding potential for cost savings in excess of 50% compared to other process configurations such as simultaneous saccharification and fermentation (SSF) or co-fermentation (SSCF) due to the elimination of the operating and capital costs associated with the additional step of enzyme production in the SSF and SSCF configurations (Olson et al., 2011). It has been estimated that the cost of ethanol produced by CBP is 4.5-fold lower than that of the SSCF configuration (Lynd et al., 2005).

CBP of lignocellulosic feedstock for H_2 production using thermophiles such as *Caldicellulosiruptor* sp. (Ivanova et al., 2009), *Clostridium* sp. (Levin et al., 2006), *Thermoanaerobacter* sp. (Shaw et al., 2010) and *Thermoanaerobacterium* sp. (Karadag et al., 2009) offers additional advantages in biomass processing over the use of mesophilic microbial systems (Mohan Raj et al., 2012). As the rate of biomass degradation increases with temperature, several techno-economical advantages to the process of H_2 can be realized through a thermophilic CBP: (1) close to theoretical maximum yield of 4 mol H_2/mol glucose; (2) utilization of a wide range of complex polymeric substrates; (3) favorable thermodynamics of stochiometric H_2 yields at higher temperatures; (4) diminished possibility of contamination by unwanted microorganisms that compete for the same substrates; (5) increased reaction/conversion rates due to improved mass transfer rates, improved substrate accessibility and solubility at elevated temperatures; (6) reduced formation of by-products; and (7) potential savings of capital and operating costs.

The extreme thermophile *Caldicellulosiruptor saccharolyticus* has been reported to produce H_2 on simple sugars such as fructose, arabinose, xylose, mannose, glucose, galactose (in descending order) (Van Fossen et al., 2009), and on a variety of polymeric substrates including agricultural waste such as wheat straw, bagasse, maize leaves (Ivanova et al., 2009), energy crops such as sweet sorghum (Ivanova et al., 2009; Panagiotopoulos et al., 2010) and *Miscanthus* (de Vrije et al., 2009), industrial waste such as paper sludge (Kadar et al., 2004), and food waste such as potato peels (Mars et al., 2010). However, no reports have been found in literature on utilization of SWG for H_2 production. The chemical composition of SWG varies with an average content of 34–39% glucan, 27–35% hemicellulose, and 19–23% lignin (Yan et al., 2010). The relatively high carbohydrate and low lignin contents makes SWG a very suitable biorefinery feedstock for bioprocessing to biofuels and biochemicals. Here we report on the direct utilization of SWG without a prior physicochemical or biological pretreatment for H_2 production by *C. saccharolyticus* strain DSM 8903.

2. Methods

2.1. Substrates and chemicals

Untreated SWG samples were a generous gift from Dr. K. Muthukumarappan from South Dakota State University, Brookings, SD. Prior to use, SWG was milled and sieved to obtain a fraction of 180 mesh uniform particle size referred to further in the text as "untreated switchgrass". MCC with an average particle size of 50 μM was purchased from Acros Organics (Thermo Fisher Scientific, NJ, USA). Yeast extract (Cat. 212750) was obtained from Difco (Becton Dickinson; Franklin Lakes, NJ, USA). Glucose and all other chemicals and reagents, unless otherwise indicated, were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Microorganism and culture conditions

The strain *C. saccharolyticus* DSM 8903 was purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) culture collection, Braunschweig, Germany. The strain was cultivated in DSM 640 culture medium with slight modifications. The culture medium had the following components per liter of deionized water: MCC, 2 g; NH_4Cl, 0.9 g; $MgCl_2{\cdot}6H_2O$, 0.4 g; NaCl, 0.9 g; $FeCl_3{\cdot}6H_2O$, 2.50 mg; K_2HPO_4, 1.5 g; KH_2PO_4, 0.75 g; yeast extract (YE), 1.0 g; cysteine-HCl, 0.75 g; resazurin, 0.5 mg; and trace element solution, 1.0 ml. The component trypticase of DSM 640 medium was replaced with casamino acid, 2.0 g/L. The trace element solution had the following components per liter of water: HCl (7.7 M), 10.00 ml; $FeCl_2{\cdot}4H_2O$, 1.50 g; $ZnCl_2$, 70.00 mg; $MnCl_2{\cdot}4H_2O$, 100.00 mg; H_3BO_3, 6.00 mg; $CoCl_2{\cdot}6H_2O$, 190.00 mg; $CuCl_2{\cdot}2H_2O$, 2.00 mg; $NiCl_2{\cdot}6H_2O$, 24.00 mg; $Na_2MoO_4{\cdot}2H_2O$, 36.00 mg. The initial pH was set at 7.2. Unless stated otherwise, the strain was grown anaerobically in 100 ml serum bottles containing 25 ml culture medium at 65 °C and 150 rpm in an orbital incubator shaker. The bottles were flushed with nitrogen gas (99.99%) for 15 min to ensure that the bottles were completely deprived of oxygen. The bottles were then sealed with butyl rubber septa and aluminum caps and autoclaved.

2.3. Determination of optimal substrates concentrations

The effects of initial SWG and glucose concentrations on the production of H_2 were studied in the range of 1–4% (w/v) in the DSM culture medium. In order to examine the effects of switchgrass and glucose, MCC of DSM medium 640 was replaced with either SWG or glucose as carbon source. The effect of yeast extract was studied in the range of 0.1–0.5% (w/v) by cultivating the strain in a modified DSM medium 640 containing 3% (w/v) SWG. In order to study the effects of insoluble SWG, the water-soluble extractives were removed from SWG by washing it in deionized water at 2% (w/v) with constant stirring at 70 °C overnight as described previously (Yang et al., 2009). The soluble extractives were filtered off and washed with deionized water twice at room temperature. The washed SWG was dried overnight at 60 °C and used in the H_2 production experiments. A seed culture of *C. saccharolyticus* DSM 8903 was routinely prepared in the modified DSM medium 640 containing 3% (w/v) SWG for 4 days. About 5 ml of a 4 day-grown culture broth was used as seed culture in all experiments. Samples were withdrawn periodically to determine cell density, residual substrate and metabolites concentration according to the methods described previously (Mohan Raj et al., 2009).

2.4. Determination of switchgrass and microbial cell morphology

In order to prepare samples for scanning electron microscopic studies (SEM), 3 ml of cell suspensions were centrifuged to obtain pellets in sterile 15 ml centrifuge tubes. After the liquid medium was discarded, 2 ml of a fixing solution (one part glutaraldehyde, nine parts of 100 mM cacodylate buffer, pH 7.2) was added to each pellet and the pellet was mixed gently. The cells were then fixed on ice for 60 min. The suspension was centrifuged at 5,000×*g* and room temperature for 10 min. The supernatant was removed with a Pasteur pipette. The cells were gradually dehydrated in 50%, 70%, 80% and 100% alcohol for 15 min. Cells were mounted on aluminum stubs and allowed to dry before subjected to microscopical observation. In order to evaluate SWG morphology, milled SWG (before fermentation) and residual SWG (after fermentation) were washed with deionized water thrice and the dried SWG particles were overlaid on aluminum stubs and observed under a Zeiss Supra40VP variable-pressure field-emission SEM.

2.5. Analytical methods

Cell concentrations were measured in a 10-mm-path-length cuvette using a double-beam spectrophotometer (Shimadzu; Columbia, MD, USA) at 580 nm. One unit of absorbance at 580 nm corresponded to 0.377 g dried cell mass per liter. However, cells that adhered on SWG, could not be measured accurately. Concentrations of glucose and fermentation metabolites were determined by HPLC (Shimadzu LC20; Columbia, MD, USA). The supernatants, obtained by centrifugation of the culture samples at 10,000×*g* for 10 min, were filtered through Nylon-membrane (Cole-Parmer; East Bunker Court Vernon Hills, IL, USA) and eluted through a 300 × 7.8 mm Aminex HPX-87H (Bio-Rad; Hercules, CA, USA) column at 60 °C using 5.0 mM H_2SO_4. The H_2 and CO_2 content in the culture media was measured using a Gas Chromatograph (Agilent 7890A; Santa Clara, CA) equipped with thermal conductivity detector (TCD) and a Porapak Q column (AW, 80–100 mesh; 6 m × 1″ × 18″). Nitrogen was used as a carrier gas at 10 ml/min. The injector, oven and detector temperatures were set at 100, 70, and 100 °C, respectively.

3. Results and discussion

3.1. Effect of substrate concentrations on cell growth and H_2 production

The effect of different concentrations of SWG (Fig. 1) and glucose (Table 1) on H_2 production by *C. saccharolyticus* DSM 8903 was examined in the range of 1–4% (w/v) in a modified DSM medium 640. According to Fig. 1, production of H_2 increased correspondingly with the increase in SWG concentrations from 1% to 3% (w/v); however, it decreased at a concentration of 4% SWG. These results indicate that strain DSM 8903 can utilize untreated SWG and produce H_2. A maximum production of 14 mmol H_2/L was accounted at 96 h with 3% SWG, indicating that this concentration was the optimal for H_2 production. To the best of our knowledge, this is the first report in literature that demonstrates high production levels of H_2 at 14 mmol H_2/L with *C. saccharolyticus* DSM 8903 on SWG without a physical, chemical or biological pretreatment.

According to Table 1, the highest H_2 production reached the theoretical maximum for dark fermentation of 4 mol H_2/mol glucose on 1% and 2% of glucose with H_2 concentrations in excess of 22 mmol H_2/L. Glucose concentrations above 2% were not favorable for growth and H_2 production. For example, on 3% glucose, both cell growth rate and H_2 production decreased (by 30%) compared to that of 2% initial glucose, while at 4% glucose the cell growth and H_2 production ceased completely, possibly due to increased osmotic pressure at higher glucose concentrations. The above results are in good agreement with those obtained by other investigators with *C. saccharolyticus* DSM 8903 (van Niel et al., 2003). The same authors also reported that growth of *C. saccharolyticus* DSM 8903 ceased completely in salt and solutes concentration between 400 and 425 mM. In our hands, however, this effect occurred at glucose concentrations of less than 200 mM. The experimental results with both SWG and glucose indicated that the ratio of H_2 to CO_2 produced was 2:1. Although the absorbance value of the SWG-grown culture broth was measured after a brief settling down of the SWG particles, the cells adhered onto the SWG residues could not be accounted. However, the cell growth measurement on glucose

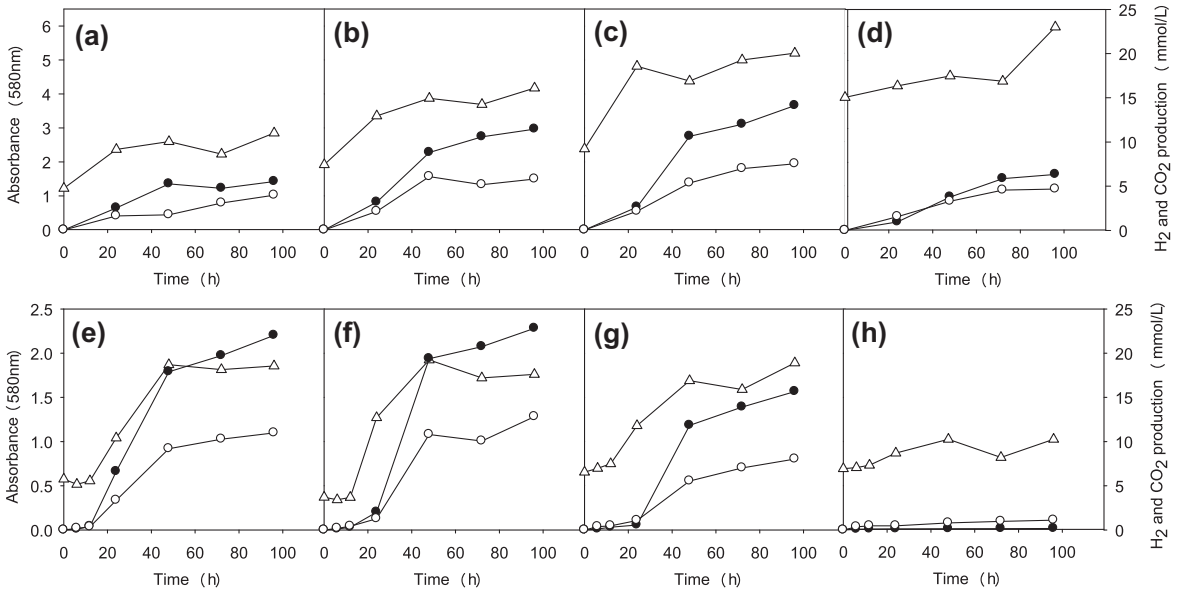

Fig. 1. Time-course of cell growth and production of H_2 and CO_2 by *C. saccharolyticus* DSM 8903 from different concentrations of switchgrass (SWG) (a–d) and glucose (e–h): (a) 1% SWG; (b) 2% SWG; (c) 3% SWG; (d) 4% SWG; (e) 1% glucose; (f) 2% glucose; (g) 3% glucose; (h) 4% glucose. Symbols: cell growth absorbance (open triangle); H_2 (closed circle); CO_2 (open circle). The values are the average of three independent sets of experiments (mean values, $n = 3$); error <5%.

Table 1
Influence of initial glucose concentration on cell growth and H_2 production by *C. saccharolyticus* DSM 8903.

Parameter	Initial glucose concentration (mM)			
	47.34	94.15	143.48	202.26
Biomass[a] (g/L)	0.52	0.48	0.47	0.12
Residual glucose[a] (mmol/L)	41.83	88.55	138.65	199.57
Glucose utilized[a] (mmol/L)	5.51	5.60	4.83	2.69
Maximum H_2 production[a] (mmol/L)	22.02	22.82	15.66	0.00
Specific growth rate[c] (/h)	0.044	0.032	0.022	0.009
Biomass yield[b] (g dcw/g glucose)	0.53	0.48	0.54	0.26
Specific H_2 production rate[d] (mmol/g dcw/h)	1.30	2.40	1.61	–
Volumetric H_2 production rate[d] (mmol/L/h)	0.47	0.72	0.47	–
H_2 yield[e] (mol/mol glucose)	4.00	4.07	3.24	–

Dcw, dry cell weight.
The values are the average of three independent sets of experiments (mean values, $n = 3$); error <5%.
[a] Accounted between 0 and 96 h.
[b] Accounted at 96 h.
[c] Calculated between 6 and 48 h.
[d] Calculated between 24 and 48 h.
[e] Calculated between 0 and 96 h.

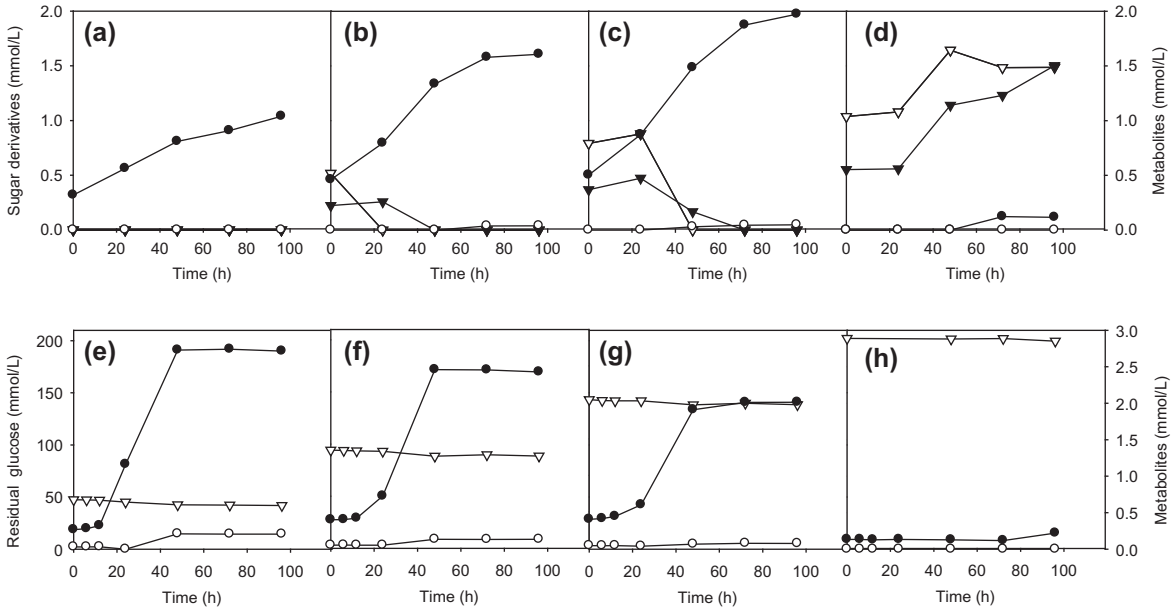

Fig. 2. Time-course of metabolic byproducts formation by *C. saccharolyticus* DSM 8903 from different concentrations of switchgrass (SWG) (a–d) and glucose (e–h): (a) 1% SWG; (b) 2% SWG; (c) 3% SWG; (d) 4% SWG; (e) 1% glucose; (f) 2% glucose; (g) 3% glucose; (h) 4% glucose. Symbols: acetate (closed circle); lactate (open circle); glucose (open triangle); xylose (closed triangle). The values are the average of three independent sets of experiments (mean values, $n = 3$); error <5%.

was accurate. In glucose medium, the cells did not grow after 48 h and was either static or approaching a decline phase after 48 h. The maximum cell density of 0.66–0.71 g dcw/L was recorded at 48 h. The cells utilized only 4.83–5.6 mM glucose in 96 h regardless of the glucose concentrations used (Table 1). The maximum glucose consumption of 5.6 mM was attained in 2% glucose after 96 h. The maximum specific growth rate (μ_{max}), estimated between 6 h and 48 h, was 0.044/h at 1% glucose, while it decreased to 0.032 and 0.022/h at 2% and 3% glucose, respectively. The biomass yield ($Y_{X/Glu}$) at 96 h was in the range of 0.48–0.54 g dcw/g glucose, which was close to the theoretical maximum of biomass yield on glucose as reported by Stephanopoulos et al. (1998).

These results suggest that *C. saccharolyticus* DSM 8903 is very efficient in conservation of energy; however, the poor glucose consumption at pH 7.2 and 65 °C is puzzling. Possible reasons for this phenomenon could be the low inoculum densities and/or limitations in one or more of the physiochemical parameters. This certainly warrants investigations of higher inoculum loadings and a thorough optimization of the fermentation parameters under controlled conditions. Although the maximum theoretical yield of 4 mol/mol glucose was achieved with 1% and 2% glucose (Table 1), the maximum specific H_2 production rate (q_{max}), calculated for the period between 24 and 48 h, was only 2.4 mmol/g dcw/h at 2% glucose, which is approximately five-fold lower than the reported value with glucose at 2 mM initial concentration (de Vrije et al., 2007). At 3% and 1% glucose, the q_{max} decreased further to 1.61 and 1.3 mmol H_2/g dcw/h, respectively. Similarly, the volumetric productivity of H_2 at 2% glucose was 0.72 mmol/L/h and only 0.47 mmol/L/h for 1% and 3% glucose. In comparison, a maximum volumetric productivity of 0.15 mmol H_2/L/h was determined for 3% SWG, which was almost five-fold lower than that obtained with 2% glucose. This suggests that although strain DSM 8903 was able to hydrolyze untreated SWG, its H_2 volumetric productivity was low, possibly due to a slow hydrolysis of SWG.

Table 2
Carbon material balance for the anaerobic metabolism of glucose by *C. saccharolyticus* DSM 8903.

Substrate and metabolites	Initial glucose concentration (mM)								
	47.34			94.15			143.48		
	mM	*C*-mM	*C* %	mM	*C*-mM	*C* %	mM	*C*-mM	*C* %
Substrate									
Glucose	5.51	33.06	100.00	5.6	33.6	100.00	4.83	28.98	100.00
Biomass	0.48[a]	17.56[b]	53.12	0.44[a]	16.10[b]	47.91	0.43[a]	15.73[b]	54.28
Metabolites									
Acetate	2.38	4.76	14.40	1.96	3.92	11.67	1.53	3.06	10.56
Lactate	0.18	0.54	1.63	0.08	0.24	0.71	0.03	0.09	0.31
CO_2	10.23	10.23	30.94	12.07	12.07	35.92	7.27	7.27	25.09
Total products	–	33.09	–	–	32.33	–	–	26.15	–
Carbon recovery[c] %	–	–	100.09	–	–	96.21	–	–	90.24

The values are the average of three independent sets of experiments (mean values, $n = 3$); error <5% for the reactant and products.
[a] g/L.
[b] Average molecular weight of 24.6 corresponding to average cell with a molecular formula of $CH_{1.8}O_{0.5}N_{0.2}$; the average ash content of 8% was deduced from the actual cell dry mass.
[c] Total carbon in biomass and metabolites ×100/total carbon in glucose.

3.2. Effect of substrate concentration on metabolite formation

During fermentation, the effect of the carbon source on the metabolite formation was also studied (Fig. 2). In both the SWG and glucose grown culture broth, more acetate was produced than lactate; however, the acetate formation on 4% SWG was insignificant, presumably due to the strain inability to utilize the SWG hydrolysis products and the poor growth metabolism. A very small quantity of lactate was produced in the glucose grown cells. This may suggest that at the above physiological conditions, disposing of electrons for maintaining the cells redox status through the H_2 production pathway was not satisfactory, and the cells must have switched to an alternative route to maintain their redox potential. In the SWG supplemented culture broth, about 0.5–1.5 mM of glucose and xylose were recorded at the beginning of fermentation. The presence of these sugars at 0 h was due to their introduction with the seed culture; the level of sugar derivatives increased with the increase in the SWG concentration (Fig. 2a–d). At 2% and 3% SWG, however, glucose and xylose could not be detected after 48 h, probably due to their utilization for cell growth and H_2 production. However, at 4% SWG, both glucose and xylose continuously accumulated in the culture broth which could be explained by a possible imbalance between the rates of saccharification and fermentation of SWG: the saccharification efficiency of *C. saccharolyticus* DSM 8903 was likely greater than its oxidative sugar metabolism.

3.3. Glucose metabolism in C. saccharolyticus DSM 8903

The carbon material balance for the anaerobic fermentation of glucose by *C. saccharolyticus* DSM 8903 was analyzed and results are summarized in Table 2. The residual metabolites profile indicate that, in addition to H_2 and CO_2, acetate and lactate were the only other end-products of *C. saccharolyticus* DSM 8903 fermentation: acetate was produced at 1.5–2.4 mmol/L, while 0.03–0.18 mmol/L of lactate was formed. The production of both metabolites was notably higher at lower glucose concentrations. The higher production of acetate at lower initial glucose concentrations could be associated with the ATP synthesis under anaerobic conditions that is required to meet the energy demand of the cell growth metabolisms. However, as evident from Table 2, the acetate to glucose ratio was fairly low (<0.5 mol acetate/mol of glucose). Similar results have been observed with *C. saccharolyticus* DSM 8903 when the cells were grown under anaerobic condition without N_2 sparging (Willquist et al., 2011). The low levels of acetate production could be the result of utilization of acetate (Ljunggren et al., 2011) and/or acetyl-CoA for cell growth metabolisms. According to the material balance data, 48–54% of the total carbon was directed towards cell biomass formation regardless of the glucose concentrations, and 25–35% of carbon was released as CO_2 during anaerobic fermentation. The remaining glucose carbon was converted to acetate and lactate. The carbon metabolites analysis showed that at 96 h of fermentation, 90–100% of the supplied glucose was recovered in the form of carbon (Table 2).

3.4. Cell morphology

The morphological changes on the surface of SWG fibers following fermentation with *C. saccharolyticus* DSM 8903 were evaluated using SEM (Supplementary Fig. 1). Before subjected to fermentation, the SWG fibers appeared smooth and the cell wall structure was clearly visible under SEM (Supplementary Fig. 1a and b). Following anaerobic fermentation, the residual SWG fibers were visibly degraded (Supplementary Fig. 1c and d). More pores were formed on the surface of the fibers, and the cell wall structures were disintegrated, which could be ascribed to enzymatic hydrolysis of SWG and solubilization of polymeric sugars. Both SWG and glucose-grown cells were evaluated for their morphology (Supplementary Fig. 1e–h). On average, the size of the cells grown in SGW was 2.7 μm in length (Supplementary Fig. 1e and g), while the size of the glucose grown cells was 4.7 μm in length (Supplementary Fig. 1f and h). This was indicative that for growth and carbon metabolisms *C. saccharolyticus* DSM 8903 favored glucose than SWG.

3.5. Effect of yeast extract on H_2 production from switchgrass

YE is used as organic nitrogen base and excellent source of amino acids, vitamins and many complex growth factors. In order to understand the effect of YE on cell growth, substrate hydrolysis and H_2 production, YE was included in the culture medium containing 3% SWG in concentrations varying from 0.1% to 0.5% (w/v). Modified DSM medium 640 containing no SWG and 0.1–0.5% YE was used as control. Fig. 3 shows the cell growth and production of H_2 and CO_2 with different concentrations of YE at the initial pH of 7.2. Although H_2 production increase correlated with the increase in YE concentrations in the control experiments (Fig. 3a–e), the influence of YE on H_2 production from SWG was insignificant. The H_2 production was in the range of 16.53–18.12 mmol H_2/L with respect to the YE concentrations (Fig. 3). A maximum volumetric productivity of 0.15 mmol/L/h was obtained at 0.5% YE. However, results suggested that YE did not improve H_2

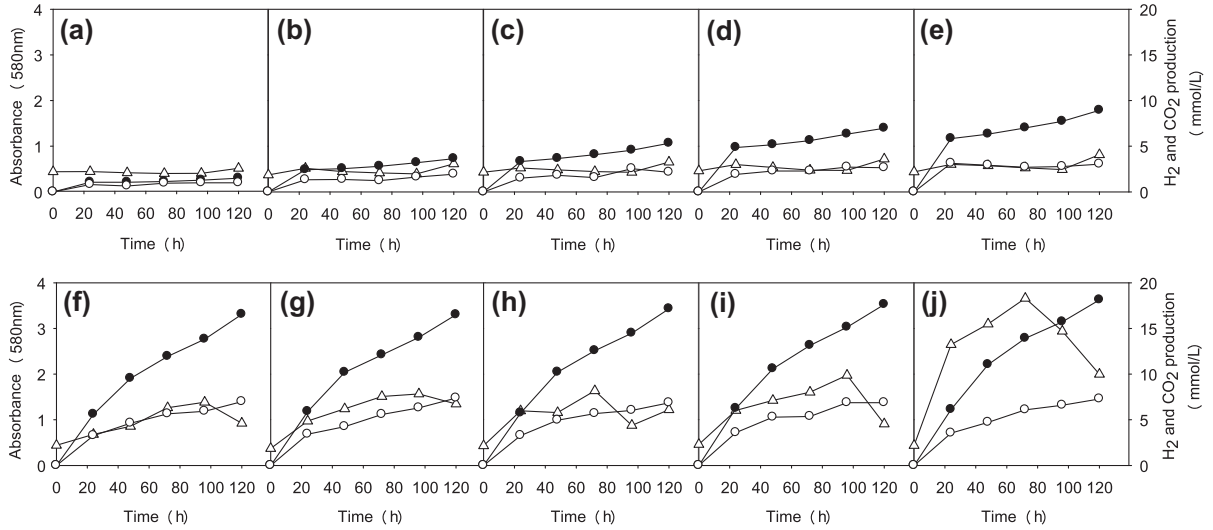

Fig. 3. Time-course of cell growth and production of H_2 and CO_2 by *C. saccharolyticus* DSM 8903 from different concentrations of yeast extract in DSM medium without switchgrass (SWG) (a–e) and DSM medium containing 3% SWG (f–j): (a) 0.1% yeast extract; (b) 0.2% yeast extract; (c) 0.3% yeast extract; (d) 0.4% yeast extract; (e) 0.5% yeast extract; (f) 0.1% yeast extract with 3% SWG; (g) 0.2% yeast extract with 3% SWG; (h) 0.3% yeast extract with 3% SWG; (i) 0.4% yeast extract with 3% SWG; (j) 0.5% yeast extract with 3% SWG. Symbols: cell growth absorbance (open triangle); H_2 (closed circle); CO_2 (open circle). The values are the average of three independent sets of experiments (mean values, $n = 3$); error <5%.

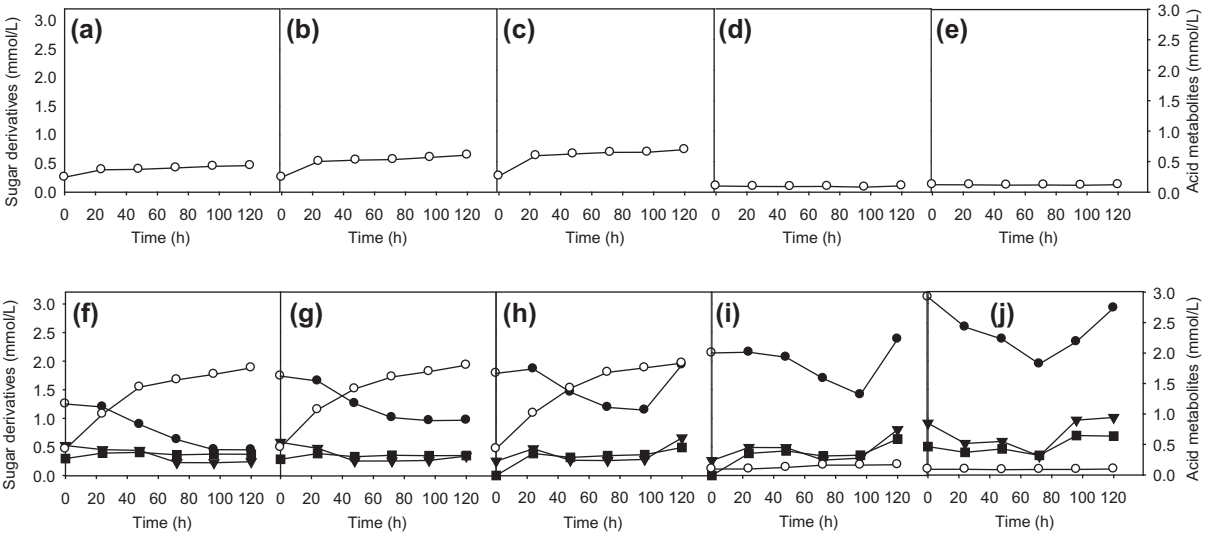

Fig. 4. Time-course of metabolites end-products formation by *C. saccharolyticus* DSM 8903 with different concentrations of yeast extract in DSM medium without switchgrass (SWG) (a–e) and DSM medium containing 3% SWG (f–j): (a) 0.1% yeast extract; (b) 0.2% yeast extract; (c) 0.3% yeast extract; (d) 0.4% yeast extract; (e) 0.5% yeast extract; (f) 0.1% yeast extract with 3% SWG; (g) 0.2% yeast extract with 3% SWG; (h) 0.3% yeast extract with 3% SWG; (i) 0.4% yeast extract with 3% SWG; (j) 0.5% yeast extract with 3% SWG. Symbols: glucose (closed circle); acetate (open circle); xylose (closed triangle); arabinose (closed square). The values are the average of three independent sets of experiments (mean values, $n = 3$); error <5%.

production. The effects of YE on the residual metabolites were analyzed for the culture broth during fermentation (Fig. 4). Acetate was formed in the culture broth at YE concentrations of 0.1–0.3% but not at 0.4% and 0.5% YE. The decreased acetate formation at higher YE concentration could be influenced by a poor metabolic flux of sugars in presence of other carbon and nitrogen rich complex nutrients. Glucose was the major carbohydrate metabolite in all SWG grown culture broths. Although some SWG-derived glucose may have been introduced with the seed culture at the time of culture inoculation, glucose was not completely utilized in the culture broths containing YE concentrations higher than 0.2%. At 0.3% and 0.4% YE, accumulation of glucose increased after 96 h, while at 0.5% YE, glucose accumulated after 72 h (Fig. 5). Xylose and arabinose were detected as degradative sugar metabolites at less than 0.5 mM, presumably introduced with the culture inoculums; however, these metabolites accumulated in the 0.3–0.5% YE supplemented culture broth after 96 h. These data may suggest the existence of different metabolic pathways in *C. saccharolyticus* DSM 8903: (1) cells did not metabolize glucose, xylose and arabinose in the presence of high concentrations of YE (a rich source of carbon, nitrogen, vitamins and amino acids supplements); (2) the higher concentrations of YE could have augmented the synthesis of hydrolytic enzymes thereby partially converting SWG to simple sugars; and/or (3) accumulation of sugars at higher YE concentrations could result from imbalances between the SWG hydrolysis and sugar fermentation rates. Overall, addition of YE did not improve the H_2 production capabilities in *C. saccharolyticus* DSM 8903.

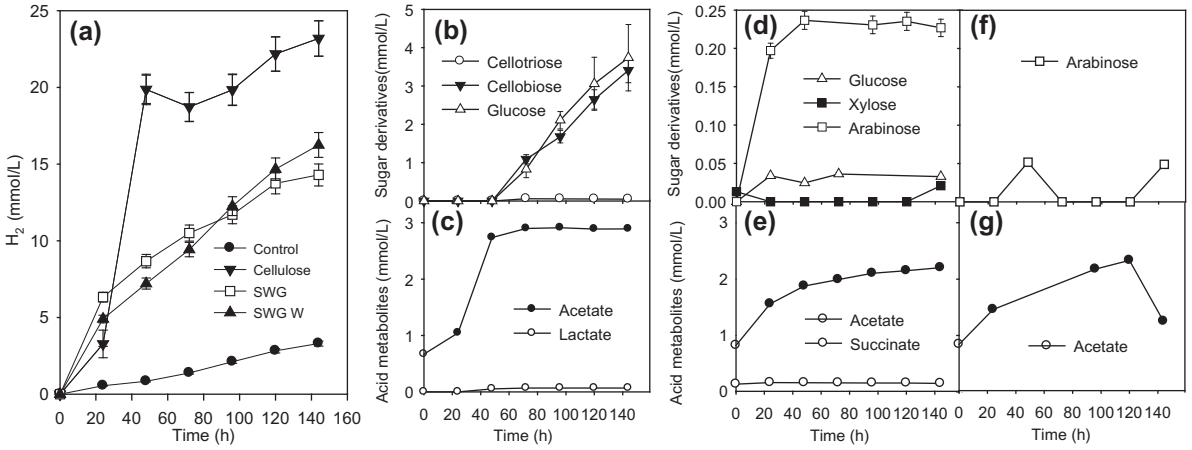

Fig. 5. Time-course of H_2 production (a) and sugar/acid metabolites formation (b–g) by *C. saccharolyticus* DSM 8903: (a) H_2 production on microcrystalline cellulose (cellulose), switchgrass-washed (SWG-washed) and switchgrass-unwashed (SWG-unwashed); (b) sugar derivatives from cellulose; (c) acid metabolites from cellulose; (d) sugar derivatives from SWG-unwashed; (e) acid metabolites from SWG-unwashed; (f) sugar derivatives from SWG-washed; (g) acid metabolites from SWG-washed. The values are the average of three independent sets of experiments (mean values, $n = 3$); error <5%.

Table 3
H_2 production by *C. saccharolyticus* DSM 8903 on polymeric substrates after 144 h.

Parameter	Polymeric substrates		
	MCC	SWG-unwashed	SWG-washed
Initial substrate (g/L)	30.00	30.00	30.00
Residual substrate (g/L)	27.53	28.89	27.88
Substrate utilized (g/L)	2.47	1.28	2.21
Maximum H_2 production (mmol/L)	23.18	14.29	16.24
Volumetric H_2 production rate (mmol/L/h)	0.16	0.10	0.11
H_2 yield (ml/g substrate)	260.49	310.33	203.44

MCC, microcrystalline cellulose.
SWG, switchgrass.
The values are the average of three independent sets of experiments (mean values, $n = 3$); error <5%.

3.6. Carbon metabolism and H_2 production capabilities on cellulose and switchgrass

In order to understand and compare the capabilities of *C. saccharolyticus* DSM 8903 for carbon metabolisms and H_2 production, two different polymeric substrates, SWG and MCC, were used at 3% (w/v) in a modified DSM medium 640 containing 0.1% YE. It should be noted that autoclaving of SWG changed the color of the culture broth from pale yellow to dark brown. The color change may be due to the release of soluble extractives (easily hydrolysable hemicellulose and pectins) from SWG. It was of interest to understand the possible role of the soluble extractives and of the insoluble residue left after their removal on H_2 production in *C. saccharolyticus* DSM 8903. After autoclaving, SWG was washed with distilled water at 70 °C overnight and the washed residue together with the control of unwashed SWG were used in the H_2 production experiments. It appeared that the difference in H_2 production between SWG-washed and SWG-unwashed after 144 h (6 d) was insignificant, suggesting that the soluble extractives released from SWG had no adverse effects on H_2 production (Fig. 5). Therefore, the ability of *C. saccharolyticus* DSM 8903 to grow on SWG was not due to the utilization of easily accessible and water-soluble extractives and sugars. This confirms published results obtained with *Anaerocellum thermophilum* DSM 6725, a close relative of *C. saccharolyticus*, on untreated (80-mesh fraction) and water-washed SWG (Yang et al., 2009). Our results show that in the case of SWG-unwashed, 1.28 g SWG/L was utilized with a maximum H_2 production of 14.3 mmol/L, yielding 11.2 mmol H_2/g SWG-unwashed biomass (Table 3). The H_2 yield after 24 h of fermentation was 6.2 mmol H_2/L (Figs. 3j and 5a). In comparison, *A. thermophilum* DSM 6725 produced less than 4.0 mmol H_2/L on SWG in 24 h (Yang et al., 2009). Ivanova et al. (2009) reported that *C. saccharolyticus* DSM 8903 preferred wheat straw over other complex lignocellulosic substrates (sweet sorghum, sugarcane bagasse, maize leaves) and produced 1.58 mmol H_2/g wheat straw. If compared to the data of the present study (11.2 mmol H_2/g SWG), this suggests that *C. saccharolyticus* DSM 8903 could produce about seven-times higher H_2 yield on SWG than wheat straw. It was also reported that acid-pretreated SWG slightly inhibited growth of *C. saccharolyticus* DSM 8903 in comparison to growth on MCC (Blumer-Schuette et al., 2010). The negative impact was ascribed to the plausible presence of sugar dehydration products and phenolics derived from the acid pretreatment of SWG. Results presented here demonstrate for first time the CBP capabilities of *C. saccharolyticus* DSM 8903 to ferment SWG to H_2 in a single step without pretreatment.

On cellulose, the highest production of 23 mmol H_2/L was obtained in 144 h, with 20 mmol H_2/L produced after 48 h; therefore, H_2 production on MCC did not increase significantly after 48 h (Fig. 5). The volumetric rate of H_2 production on cellulose, estimated between 24 and 48 h, was 0.7 mmol/L h, which represents an almost seven-fold increase over the rate obtained on SWG (both washed and unwashed) between 24 and 48 h (0.097 mmol/L h). Therefore, hydrolysis of MCC by *C. saccharolyticus* DSM 8903 was

much faster than hydrolysis of SWG. Based on a total H_2 production of 23.18 mmol/L and an uptake of 2.47 g MCC/L, the yield on MCC was 9.4 mmol H_2/g MCC.

Acetate was the major organic acid end-product of *C. saccharolyticus* DSM 8903 fermentation on both SWG and cellulose (Fig. 5b–g) whereas lactate was produced in negligible quantities. In contrast, lactate was the major metabolic end-product from the growth of *A. thermophilum* DSM 6725 on SWG and cellulose (Yang et al., 2009), suggesting a significant difference between the carbon metabolisms of these two closely related organisms. Cellobiose and glucose were produced at up to 4 mM in the culture broth supplemented with MCC during anaerobic fermentation (Fig. 5b). Cellopentose and cellotriose were also detected in trace quantities after 72 h of fermentation. In the SWG (washed and unwashed) supplemented culture broth, arabinose and glucose were also detected. In the culture grown on SWG-unwashed, glucose at 0.05 mM and arabinose up to 0.25 mM were formed (Fig. 5d). Xylose was also detected at 144 h of fermentation. In comparison, the hydrolysis products released from SWG by *A. thermophilum* DSM 6725 were glucose, cellobiose, cellotriose, galactose, xylose and xylobiose, while only glucose in trace amounts (<0.1 mM) was detected when untreated poplar biomass was used as a carbon and energy source (Yang et al., 2009).

The gradual accumulation of cellobiose in the MCC supplemented culture medium indicates poor cellobiase (β-glucosidase, and cellobiose phosphorylase) activity. However, the cellulase (cellulose 1,4-β-cellobiosidase/exoglucanase) activity responsible for cellobiose formation was efficient in this strain even after fermentation ceased. The glucose accumulation after 48 h could be the result of a pH shift that influenced the cell growth and metabolism in *C. saccharolyticus* DSM 8903.

4. Conclusions

The H_2 production capabilities of the extreme thermophile *C. saccharolyticus* DSM 8903 were examined on untreated SWG, MCC and glucose. It was demonstrated that *C. saccharolyticus* can: ferment SWG at 11.2 mmol H_2/g of SWG without any physico-chemical or biological pretreatment to H_2 in a single step; ferment MCC at 9.4 mmol H_2/g cellulose at a seven-fold higher H_2 production rate than SWG; reach the theoretical yield of 4 mol H_2/mol glucose. Further in-depth investigations of the redox and energy metabolism coupled with strain improvement strategies are needed to optimize *C. saccharolyticus* as the ultimate CBP organism for large-scale H_2 production.

Acknowledgements

Financial support by the Center for Bioprocessing Research and Development (CBRD) at the South Dakota School of Mines & Technology (SDSM&T), the South Dakota Board of Reagents (SD BOR), the South Dakota Governor's Office for Economic Development (SD GOED), and the U.S. Air Force Research Laboratory (AFRL) is gratefully acknowledged.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2013.04.005.

References

Bielen, A.A.M., Verhaart, M.R.A., van der Oost, J., Kengen, S.W.M., 2013. Biohydrogen production by the thermophilic bacterium *Caldicellulosiruptor saccharolyticus*: current status and perspectives. Life 3, 52–85.

Blumer-Schuette, S.E., Lewis, D.L., Kelly, R.M., 2010. Phylogenetic, microbiological, and glycoside hydrolase diversities within the extremely thermophilic, plant biomass-degrading genus *Caldicellulosiruptor*. Appl. Environ. Microbiol. 76, 8084–8092.

de Vrije, T., Mars, A.E., Budde, M.A.W., Lai, M.H., Dijkema, C., Waard, P., Claassen, P.A.M., 2007. Glycolytic pathway and hydrogen yield studies of the extreme thermophile *Caldicellulosiruptor saccharolyticus*. Appl. Microbiol. Biotechnol. 74, 1358–1367.

de Vrije, T., Bakker, R.R., Budde, M.A.W., Lai, M.H., Mars, A.E., Claassen, P.A.M., 2009. Efficient hydrogen production from the lignocellulosic energy crop *Miscanthus* by the extreme thermophilic bacteria *Caldicellulosiruptor saccharolyticus* and *Thermotoga neapolitana*. Biotechnol. Biofuels 2, 1–15.

Ivanova, G., Rakhely, G., Kovacs, K.L., 2009. Thermophilic biohydrogen production from energy plants by *Caldicellulosiruptor saccharolyticus* and comparison with related studies. Int. J. Hydrogen Energy 34, 3659–3670.

Kadar, Z., de Vrije, T., van Noorden, G.E., Budde, M.A., Szengyel, Z., Reczey, K., Claassen, P.A., 2004. Yields from glucose, xylose, and paper sludge hydrolysate during hydrogen production by the extreme thermophile *Caldicellulosiruptor saccharolyticus*. Appl. Biochem. Biotechnol. 113–116, 497–508.

Karadag, D., Mäkinen, A.E., Efimova, E., Puhakka, J.A., 2009. Thermophilic biohydrogen production by an aerobic heat treated-hot spring culture. Bioresour. Technol. 100, 5790–5795.

Levin, D.B., Islam, R., Cicek, N., Sparling, R., 2006. Hydrogen production by *Clostridium thermocellum* 27,405 from cellulosic biomass substrates. Int. J. Hydrogen Energy 31, 1496–1503.

Ljunggren, M., Willquist, K., Zacchi, G., van Niel, E.W.J., 2011. A kinetic model for quantitative evaluation of the effect of hydrogen and osmolarity on hydrogen production by *Caldicellulosiruptor saccharolyticus*. Biotechnol. Biofuels 4, 31.

Lynd, L.R., van Zyl, W.H., McBride, J.E., Laser, M., 2005. Consolidated bioprocessing of cellulosic biomass: an update. Curr. Opin. Biotechnol. 16, 577–583.

Mars, A.E., Veuskens, T., Budde, M.A.W., van Doeveren, P.F.N.M., Lips, S.J., Bakker, R.R., de Vrije, T., Claassen, P.A.M., 2010. Biohydrogen production from untreated and hydrolyzed potato steam peels by the extreme thermophiles *Caldicellulosiruptor saccharolyticus* and *Thermotoga neapolitana*. Int. J. Hydrogen Energy 35, 7730–7737.

Mohan Raj, S., Rathnasingh, C., Jung, W.C., Park, S., 2009. Effect of process parameters on 3-hydroxypropionic acid production from glycerol using a recombinant *Escherichia coli*. Appl. Microbiol. Biotechnol. 84, 649–657.

Mohan Raj, S., Talluri, S., Christopher, L.P., 2012. Thermophilic hydrogen production: current status and future perspectives. Bioenergy Res. 5, 515–531.

Olson, D.G., McBride, J.E., Shaw, A.J., Lynd, L.R., 2011. Recent progress in consolidated bioprocessing. Curr. Opin. Biotechnol. 23, 1–10.

Panagiotopoulos, I.A., Bakker, R.R., de Vrije, T., Koukios, E.G., Claassen, P.A.M., 2010. Pretreatment of sweet sorghum bagasse for hydrogen production by *Caldicellulosiruptor saccharolyticus*. Int. J. Hydrogen Energy 35, 7738–7747.

Shaw, A.J., Hogsett, D.A., Lynd, L.R., 2010. Natural competence in *Thermoanaerobacter* and *Thermoanaerobacterium* species. Appl. Environ. Microbiol. 76, 4713–4719.

Stephanopoulos, G.N., Aristidou, A.A., Nielsen, J., 1998. Metabolic Engineering: Principles and Methodologies, first ed. Academic Press, San Diego.

Van Fossen, A.L., Verhaart, M.R., Kengen, S.W.M., Kelly, R.M., 2009. Carbohydrate utilization patterns for the extremely thermophilic bacterium *Caldicellulosiruptor saccharolyticus* reveal broad growth substrate preferences. Appl. Environ. Microbiol. 25, 7718–7724.

van Niel, E.W.J., Claassen, P.A.M., Stams, A.J.M., 2003. Substrate and product inhibition of hydrogen production by the extreme thermophile, *Caldicellulosiruptor saccharolyticus*. Biotechnol. Bioeng. 81, 255–262.

Willquist, K., Pawar, S.S., van Niel, E.W.J., 2011. Reassessment of hydrogen tolerance in *Caldicellulosiruptor saccharolyticus*. Microb. Cell Fact. 10, 111.

Wooley, R., Ruth, M., Glassner, D., Sheehan, J., 1999. Process design and costing of bioethanol technology: a tool for determining the status and direction of research and development. Biotechnol. Progr. 15, 794–803.

Yan, J., Zhoujian Hu, Z., Pu, Y., Brummer, E.C., Ragauskas, A.J., 2010. Chemical compositions of four switchgrass populations. Biomass Bioenergy 34, 48–53.

Yang, B., Wyman, C.E., 2008. Pretreatment: the key to unlocking low-cost cellulosic ethanol. Biofuels, Bioprod. Biorefin. 2, 26–40.

Yang, S.J., Kataeva, I., Hamilton-Brehm, S.D., Engle, N.L., Tschaplinski, T.J., Doeppke, C., Davis, M., Westpheling, J., Adams, M.W.W., 2009. Efficient degradation of lignocellulosic plant biomass, without pretreatment, by the thermophilic anaerobe *Anaerocellum thermophilum* DSM 6725. Appl. Environ. Microbiol. 75, 4762–4769.

Zheng, Y., Yu, X., Zeng, J., Chen, S., 2012. Feasibility of filamentous fungi for biofuel production using hydrolysate from dilute sulfuric acid pretreatment of wheat straw. Biotechnol. Biofuels 5, 50.